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Diagnosis of Ebola Haemorrhagic Fever by RT-PCR in an Epidemic Setting

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This study reports the first field evaluation of a new diagnostic technique for Ebola virus disease with sensitivity and specificity. Ebola virus causes rare but fulminating outbreaks in Equatorial Africa. Rapid differentiation from other infections is critical for timely implementation of public health measures. Patients usually die before developing antibodies, necessitating rapid virus detection. A reverse transcriptase-polymerase chain reaction (RT-PCR) assay was developed, implemented and evaluated at Centre International de Recherches Médicales de Franceville (CIRMF) in Gabon, to detect Ebola viral RNA in peripheral blood mononuclear cells (PBMC). Twenty-six laboratory-confirmed patients during and 5 after the acute phase of Ebola haemorrhagic fever, 15 healthy controls and 20 febrile patients not infected with Ebola virus were studied. RT-PCR results were compared with ELISA antigen capture, and Ebola specific IgM and IgG antibody detection. Ebola virus RNA was amplified from 26/26 specimens from the acute phase, 3/5 during recovery, 0/20 febrile patients and 1/15 negative controls. Sensitivity of RT-PCR in identifying acute infection and early convalescence compared with antigen or IgM detection was 100% and 91% respectively, and specificity compared with antigen detection and IgM assay combined was 97%. Antigen capture detected only 83% of those identified by PCR, and IgM only 67%. Ebola virus RNA was detected in all 13 fatalities, only 5 of whom had IgM and none IgG. RT-PCR detected Ebola RNA in PBMC one to three weeks after disappearance of symptoms when antigen was undetectable. RT-PCR was the most sensitive method and able to detect virus from early acute disease throughout early recovery. ***J. Med. Virol. 60:463–467, 2000.***

KEY WORDS: filovirus; Africa; antigen; antibody

INTRODUCTION

Two outbreaks of Ebola haemorrhagic fever in Gabon in early (Mayibout) and late (Booué) 1996 resulted in mortality of 66% and 75% respectively in laboratory-confirmed patients [Georges-Courbot et al., 1997; Georges et al., 1999]. Because of the rapid disease course, high mortality, and transmissibility both in the community and in hospitals, immediate diagnosis is essential for institution of appropriate surveillance and control. The overriding requirement is a rapid test with very high sensitivity, because the consequences of a false negative can be extremely grave.

Ebola virus is an enveloped, non-segmented negative-strand RNA virus, belonging to the *Filoviridae* family. Several diagnostic assays for Ebola infection are currently used and have been recently evaluated in patients in the field during the Kikwit outbreak [Ksiazek et al., 1999a,b]. Detection of Ebola antigens or Ebola virus specific IgM in the peripheral circulation may be realised by ELISA, but neither test is sufficiently sensitive for all stages of the disease and in any event specific reagents are not generally available in countries endemic for Ebola virus. Virus isolation, the most definitive but not necessarily the most sensitive method, requires high level biocontainment, and regardless is too slow for outbreak detection and management. Similarly electronmicroscopy and histological techniques are sensitive methods particulary reliable for post-mortem diagnosis [Zaki et al., 1999], but require sophisticated equipment and reagents and are also not suitable for outbreak management in Africa.

Faced with experiences detecting Ebola epidemics in Gabon, a single test more sensitive and reliable than

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the existing techniques found unsatisfactory in practice in the field is required. The increasing ability of many laboratories in Africa to perform relatively sophisticated techniques now makes judicious application of modern techniques a realistic proposition. Many countries are now increasingly reluctant to permit exportation of specimens, and in any event cannot afford to wait for results. Hence, in addition to existing techniques a reverse transcriptase-polymerase chain reaction (RT-PCR) assay, using reagents easily prepared, was developed in a laboratory a few hours by road from an epidemic site. RT-PCR has the added advantage of requiring potentially virucidal reagents (e.g., guanidinium) and early heating steps thus enhancing laboratory safety. As peripheral blood mononuclear cells (PBMC) are known to be targets for filovirus [Feldmann et al., 1996], PBMC were probed to increase sensitivity. A retrospective diagnostic technique is also required, because Central African villages are often deep in the rainforest and intervention of medical teams can be delayed.

MATERIALS AND METHODS

Study Population

Sixty-six subjects in four groups, 31 of whom had clinically and laboratory confirmed Ebola infection: i) 26 during the acute phase of Ebola haemorrhagic fever, 24 of whom were positive by Ebola antigen detection at the time the RT-PCR sample was taken; ii) 5 patients previously acutely ill studied during the early recovery phase; iii)15 healthy subjects (endemic controls), living in the outbreak area but with no contact with symptomatic Ebola-infected patients; and iv) 20 patients (febrile controls) with high fevers negative for Ebola virus antigen and antibody (10 malaria and 10 gastrointestinal infections, all laboratory confirmed), who did not live in the endemic area and did not subsequently develop antibodies to Ebola virus.

Sample Preparation

Verbal consent was obtained, and whole blood was collected into 5 ml vacutainers containing EDTA and transported on ice to the Centre International de Recherches Médicales de Franceville (CIRMF). Potentially infected specimens were drawn and manipulated according to WHO guidelines on viral haemorrhagic fever agents in Africa [WHO, 1985]. PBMC were separated from whole blood by using Ficoll-diatrizoate (Pharmacia, Uppsala, Sweden) density centrifugation. Sera and plasma were stored at –80°C for antibody and antigen studies. RNA was extracted from PBMC by using an RNA extraction kit (RNeasy®, Qiagen, Hilden, Germany), aliquoted and stored at –80°C. RNAase free plastic labware and water were used for all procedures.

Enzyme Linked Immunosorbent Assays

Sera were tested for the presence of Ebola viral antigens using a sandwich enzyme immunosorbent assay [Ksiazek et al., 1992, 1999a] and by standard ELISA [Ksiazek et al., 1999b] for Ebola virus-specific IgG at CIRMF using reagents kindly provided by the Special Pathogens Branch, Centers for Disease Control (CDC), Atlanta. Ebola virus-specific IgM was evaluated by antibody-capture ELISA (kindly carried out by the Special Pathogen Branch, CDC).

Virus Isolation

Vero-E6 cells in 25 cm^2 flasks were used for culture and isolation of Ebola virus in the sera of 3 fatalities and 3 patients who survived, in specimens taken during the acute phase of the disease. Primary isolation in tissue culture was attempted without blind passage, and this was not done until 1998 when a laboratory with biosafety level 4 cabinet characteristics became available in Franceville.

Reverse Transcription and Polymerase Chain Reaction

Reverse transcription of RNA was undertaken using 10 μl of material in 20-μl final volume containing 1.3 μl of RNAase free water, 4 μl of 5× reverse transcription buffer (Gibco BRL, Cergy-Pontoise, France), 0.5 μl of RNAase inhibitor (Boehringer, Mannheim, Germany), 5mM dithiothreitol (Gibco BRL), 1 μM random hexamer primers (Boerhinger), 500 μM of each dATP, dCTP, dGTP, dTTP (USB, Amersham, Les Ulis, France), and 200 U of reverse transcriptase (Gibco BRL). The reaction mixture was incubated at 42°C for 90 min then heated to 65°C for 5 min to denature the reverse transcriptase. One fifth of the first-strand cDNA solution was then amplified in a 50-μl volume using 38 μl of RNAase free water, 5 μl of 10× PCR buffer (Appligene, Illrich, France), 0.5 U *Taq* polymerase, 200 μM of each deoxynucleoside triphosphate (Amersham) and 0.4 μM primers from a conserved region of the L gene coding for the polymerase determined from Zaire strain sequences [Sanchez et al., 1999] 5′-ATCGGAATTTTCTTTCTCATTGAAAGA-3′ and 5′-ATGTGGTGGATTATAATAATCACTGACATGCAT-3′.

PCR was carried out for 40 cycles (Perkin Elmer, Paris, France), each consisting of 94°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec, leading to amplification of a 420 base pair (bp) fragment. Both positive and negative controls were included in each run to confirm that only cDNA PCR products were detected and that no contamination with cDNA or previous PCR products had occurred. The amplification products were analysed by electrophoresis in 1.5% agarose gel and visualised under UV illumination after staining with ethidium bromide.

Sequencing of the Fragment

The PCR product was excised from the gel, extracted, and purified using an acid phenol-chloroform protocol. Sequencing was carried out directly using Sequenase II

kit (Amersham, Les Ulis, France) according to the manufacturer's instructions.

DNA Labelling Reaction

Amplification products were electrophoresed in 2% Nusieve agarose gels (Sigma, La Verpillière, France) and stained with ethidium bromide. The 420-bp product was then excised, extracted from the gel, purified using an acid phenol-chloroform protocol. The 420-bp fragments were extracted twice with acid phenol, then chloroform, and precipitated twice in chilled ethanol before resuspension in sterile water. Digoxigenin labelling reaction was carried out according to protocol (Boehringer).

Southern Blot Hybridisation Using Chemiluminescent Detection

Amplified products were stained in 1.5% agarose gels and transferred overnight onto a positively charged nylon membrane (Amersham) with 1 M NaOH buffer. Hybridisation and chemiluminescent detection were carried out according to protocol (Boehringer). The membrane was prehybridised in a solution containing 50mM Tris, 1M Nacl, 1% Sodium Docedyl Sulphate (SDS), 2% blocking reagent, pH 7.5, at 65°C for 2 hr, and hybridised in prehybridisation solution containing 5% sulphate dextran and 10 ng/ml of digoxigenin-labelled probe at 65°C, overnight. Membranes were pre-treated with the blocking reagent, then incubated with a dilution of anti-digoxigenin Fab fragments, then incubated with CSPD and exposed for 30 min to X-ray film.

Analysis of Data

For the purpose of estimations of sensitivity and specificity the 31 acute and recovering patients were considered to have had current or recent confirmed Ebola infection, because all 31 had had clinically compatible disease and were at some stage ELISA antigen or IgM positive. The traditional 'gold standard' for such analyses is virus isolation. This was not done for all patients because of safety considerations, and in any event, it is unclear whether isolation is more or less sensitive than modern assays such as RT-PCR. Consequently, the sensitivity of each test was calculated by its ability to identify a blood specimen as being from a patient acutely infected by Ebola virus, compared with another assay as the standard. That is, using ELISA antigen detection or IgM assay as the standard, RT-PCR was evaluated; and conversely using RT-PCR detection as the standard, ELISA antigen or IgM detection were evaluated. Specificity of the assays, that is the ability of the assays to correctly identify specimens from patients with fever due to other pathogens or from healthy individuals, were also calculated. Independence of each sample was assumed when calculating sensitivity and specificity. IgG was not evaluated as a means of detecting acute or current infections.

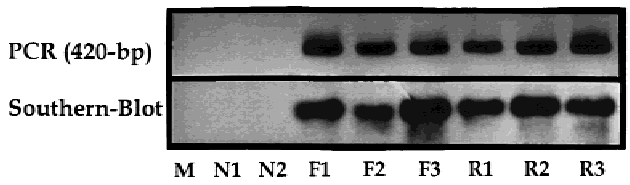

Fig. 1. PCR and Southern blot analyses of patient samples. M, PCR mix without cDNA; N, negative controls; F, fatal outcome patients during symptomatic phase; R, recovery outcome patients during symptomatic phase. All these fragments were sequenced, and were found Ebola virus specific.

RESULTS

Polymerase Chain Reaction and Hybridisation Techniques

Figure 1 shows amplified cDNA fragments on an agarose gel after staining with ethidium bromide and after probing with a digoxigenin-labelled probe. The specimens shown in this figure were from 2 negative controls and 6 symptomatic patients (3 fatal outcome, 3 non fatal outcome patients). The appropriate 420-bp fragment is detected in all symptomatic patients. Sequence analysis revealed highly EBO-Zaire-related sequences (99% homology) in these symptomatic patients, indicating that this fragment is specific for Ebola virus.

Analysis of Patient Data

Data from four different diagnostic techniques carried out on specimens from 66 different individuals are summarised in Table I. Circulating Ebola antigens were detected by ELISA techniques in 24 of 26 patients bled during the symptomatic phase. The two negative samples were taken at the onset of symptoms, thus very early in the disease process. Antigen was only detected in one of the five patients bled during the recovery phase, and then just only one day after disappearance of symptoms. Specific IgM was detected in 17/26 symptomatic patients (12 survivors and 5 fatalities) and IgG was detected in 12/26 (all survivors). Specific antibodies (IgM and IgG) were present in all 5 patients during recovery.

All of the 26 symptomatic patients were positive for RT-PCR, and unexpectedly, 3/5 patients were positive during convalescence (one, two and three weeks after disappearance of symptoms). The two specimens from Ebola patients negative by RT-PCR, were both taken one month after resolution of symptoms. No RT-PCR amplification fragment was detected in any of the specimens from the 20 febrile controls, but did in a sample from one of the healthy endemic controls. It is certainly a false positive from non-specific amplification, because this individual had no disease, was negative for Ebola antigen and never developed any antibody response to Ebola virus.

Virus Isolation

Ebola virus was isolated retrospectively on Vero-E6 cells from stored serum from 3 fatalities from the 1996

TABLE I. Results of RT-PCR, ELISA Antigen Detection and IgM Assays

Group	Antigen detection	IgM detection	RT-PCR
Acute phase (n = 26)	24	17	26
Survivors (n = 13)	12	12	13
Fatalities (n = 13)	12	5	13
Recovery (n = 5)	1	5	3
Healthy (n = 15)	0	0	1
Febrile patients (n = 20)	0	0	0

outbreaks, but virus was not isolated from serum from 3 survivors at least without blind passages.

Estimations of Sensitivity and Specificity

Table II shows that RT-PCR assay has the highest probability of detecting Ebola virus in a patient with current or recent active infection. Sensitivity of RT-PCR is 100% compared to ELISA antigen detection, and 91% compared to IgM detection. Among the 30 patients with Ebola RNA detected by RT-PCR, the ELISA antigen detection had a sensitivity of 83%, and the IgM assay 67%. Specificity of RT-PCR was calculated to be 97%. Ninety-five percent confidence intervals are found in Table II.

DISCUSSION

These data provide evaluation of a test for early and accurate detection of Ebola virus infection that may be applied widely. Diagnostic by RT-PCR can now be carried out in many laboratories in a number of developing countries in Africa, and should no longer be considered the exclusive province of developed countries. Ebola haemorrhagic fever can be difficult to distinguish clinically in early disease from other common tropical infections, such as *Shigella* dysentery, yellow fever, typhoid or malaria. Differentiation is critical because yellow fever can be controlled by vaccination, and case-to-case spread is not reported, and bacterial and parasitic infections require specific therapy. Ebola infection, with a rapidly evolving clinical course, has a very high mortality, and elevated risk of hospital and community transmission. There is neither vaccine nor treatment. Accurate and prompt diagnosis is essential to instituting strict control measures to prevent spread and proper supportive care to limit mortality. Specificity though desirable is of lesser concern. The public health consequences of failure to institute immediate control measures may result in a catastrophic epidemic. The consequences of a false positive identification are primarily delay in making a correct diagnosis and inconvenience.

Recent field evaluation during the Kikwit outbreak in Republic Democratic of the Congo has shown that current diagnostic techniques for Ebola virus infection have limitations [Ksiazek et al., 1999]. In this last study IgG and IgM appeared as late as 8–10 days after disease onset and were not always detected before death. An another study during the 1996 Gabon outbreaks has recently shown that specific IgM were observed in only one-third at modest levels and IgG were never detected in fatal cases [Baize et al., 1999]. In both patients of Kikwit and Gabon outbreaks viral antigen load in blood was correlated with symptoms, and no antigen was detected during incubation period and during convalescence. In this present study the ELISA antigen assay does not detect virus as early as PCR, and only detected cases during the symptomatic phase. Antibody assays are most useful after resolution of symptoms where IgG and IgM were always detected. Furthermore, absence of specific reagents for these tests, such as Ebola antigens and polyclonal anti-Ebola antibodies, make them generally unavailable where they are most needed. Virus isolation even given the small BSL-4 laboratory now available at CIRMF is less safe and much less practicable during epidemics, and takes too long. Furthermore, the biology and thus ease of isolation of a given strain may very well not be known at the beginning of an epidemic. It is worth remembering that Ebola virus from Sudan is particularly difficult to grow in primary isolation in tissue culture, and may require blind passages [McCormick et al., 1983].

These and other logistic constraints prompted the development and the evaluation of a RT-PCR-based method for the detection of Ebola-virus RNA in PBMC of patients infected with Ebola virus. PBMC were preferred than plasma as source of material because monocytes can be infected in vitro by filoviruses [Feldmann et al., 1996] and because no virus RNA was amplified in plasma from symptomatic patients who survived, either during symptoms and during convalescence [Leroy and Baize, unpublished data]. By contrast, viral RNA could be detected in PBMC for up to three weeks after infection.

Ebola virus RNA was detected very early in infection, before the antigen, IgM and IgG tests were positive, already providing a substantial advantage over other assays. Samples taken during the incubation phase were not available, but it may be possible to perform precocious diagnosis of Ebola infection by RT-PCR. This would be highly useful during an epidemic, when decisions are made leading to interruption of transmission. Conserving the precious time of medical personnel is also important. Infected patients must be isolated and barrier nursed if ill, and contacts must be placed under surveillance.

In view of known high level of genetic stability of filovirus especially in the L gene [Sanchez et al., 1993], primers from Ebola virus Zaire-strain sequences in L gene were preferentially designed [Sanchez et al., 1999]. The nucleotide sequence of the Gabon strain was not available when this study started, but recently, the GP and VP24 gene sequences from Gabon isolates have in fact been shown to be extremely close to those of the Zaire strain [Volchkov et al., 1997]. Thus, this assay would also be able to detect Ebola virus infections in other parts of Central or West Africa.

It is important to use classical analyses to derive estimates of sensitivity and specificity of the assay.

TABLE II. Analysis of Specificity and Sensitivity of RT-PCR

	Antigen detection	IgM detection	RT-PCR
Sensitivity of each test relative to RT-PCR as standard	83% (25/30) (95% CI 65.3–94.4)	67% (20/30) (95% CI 47.2–82.7)	100% (30/30) (95% CI 88.4–100)
Sensitivity of RT-PCR relative to other tests (antigen detection or IgM assay as standard)	100% (25/25) (95% CI 86.3–100)	90.9% (20/22) (95% CI 70.8–98.9)	93.5% (29/31) (95% CI 78.6–99.2)
Specificity of each test	100% (35/35) (95% CI 89.9–100)	100% (35/35) (95% CI 89.9–100)	97.1% (34/35) (95% CI 85.1–99.9)

Specificity (97%) was excellent, essentially equal to antigen capture or IgM detection. The sensitivity of RT-PCR was substantially higher (100%) when antigen detection was used as the standard, and 91% when the IgM was the standard. The lesser value using IgM is explained by the fact that the 2 patients who were IgM+ but RT-PCR– were sampled around one month after the disappearance of symptoms. For about 3 weeks after disappearance of symptoms, the sensitivity of RT-PCR still remains 100% with the IgM as the standard. By comparison antigen capture ELISA has a sensitivity of 83%, and IgM only 67% using RT-PCR as the standard. With these stringent criteria of evaluation, the performance of the RT-PCR was superior to the other methods. This essential approach to evaluation of diagnostic assays using well characterised patient specimens has been largely ignored in published accounts of haemorrhagic fever diagnostic techniques, many of which are described loosely, but incorrectly, as 'classical' or 'standard.'

With this said there are some caveats. Care should be taken to understand the circumstances in which the RT-PCR is carried out, the level of skill and the reproducibility of a given laboratory. There is always concern about 'false positives' due to laboratory contamination with amplicons. This problem is likely to be much less important in field laboratories where very limited molecular virology (if any) is carried out and 'amplicon density' normally very low. In contrast, in the endemic situation, failure to identify a potential case rapidly has much more serious consequences than misidentifying.

Despite these obvious obstacles, the development and eventual use of RT-PCR for diagnosis of Ebola infection in African laboratories is to be recommended. This study shows that RT-PCR assay is a powerful tool for a rapid, reliable and low-cost diagnosis of Ebola infection. It can be established in selected laboratories in Africa and elsewhere without the need of highly specialised reagents or specific structures such as BSL-4 biocontainment. Careful handling of specimens and the use of chemical reagents with virucidal action (EDTA, guanidinium) limit laboratory risk. Rapid diagnosis allows public health authorities in equatorial Africa to identify, isolate and care for patients quickly and effectively. Back-up laboratories elsewhere can follow through with confirmatory tests. Swift, specific public health intervention is the best provision currently available to reduce the toll of this much-feared disease.

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